REMARKS

The Applicant notes with appreciation the withdrawal of prior objections and rejections.

Claims 11-14, 16-18, 22-23 and 28 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Hodgson. The Applicant notes the Examiner's detailed comments concerning the hypothetical application of Hodgson to those claims. The Applicant respectfully submits that those claims are allowable over Hodgson for the reasons set forth below.

First, the Applicant notes that a claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described in a single prior art reference. The identical invention must be shown in as complete detail as is contained in the claim. There must be no difference between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention.

In this case, independent Claims 11 and 28 are directed to a method for preparing circularized recombinant nucleic acids from a vector and an insert. Claim 11 recites: "A method for preparing circularized recombinant nucleic acids from a vector and an insert comprising the steps of: producing circularized recombinant nucleic acid by ligating a DNA insert and a DNA vector in the presence of a DNA compaction agent . . . and selecting said circularized recombinant nucleic acid, **wherein said DNA compaction agent is present at a concentration sufficient to allow the DNA insert to remain flexible** . . . "

Claim 28 recites: "A method for preparing circularized recombinant nucleic acids from a vector and an insert comprising the steps of: producing circularized recombinant nucleic acid by ligating a DNA insert and a DNA vector in the presence of a DNA compaction agent . . . and selecting said circularized recombinant nucleic acid, wherein **said DNA compaction agent is present at a concentration sufficient to allow the DNA insert to remain flexible.**"

In sharp contrast, Hodgson generally discloses a method that uses compacting agent to condense the ligated DNA so that the DNA can be physically moved by pipette with reduced shearing (col. 23, lines 49-53). Hodgson, however, does not disclose that the "DNA compaction agent is present at a concentration sufficient to allow the DNA insert to remain flexible," as recited in Claims 11 and 28.

The rejection alleges that "DNA condensed on a histone protein is not rigid there is no evidence the DNA insert does not retain flexibility." (Office Action, page 5, lines 18-19). The Applicant respectfully submits that the record does not support that position. Instead, the Applicant respectfully submits that the record supports something quite different.

It was well-known to one skilled in the art at the time of the invention that binding of histone or histone-like protein to a DNA molecule reduces DNA flexibility. The Applicant has attached Zu et al., *J. Bacterol.* 178:2982-2985 (1996) (BpH1 binding to DNA fragment diminished flexibility of the DNA fragment) and Cam E.L. et al., *J. Mol. Biol.* 285:1011-1021 (1999) (binding of histone-like protein MC1 to DNA reduces the flexibility of the DNA). While the presence of a compacting agent may help to bring together the two ends of a finished vector to facilitate ligation, the compacting agent at a high concentration may also cause rigidification of the DNA. If rigidification takes place, the DNA cannot bend, and there will not be any contact between the ends of the finished vector and ligation will be impeded as described in the Applicant's Specification at paragraph 33. Therefore, the compacting agent of Claims 11 and 28 "is present at a concentration sufficient to allow the DNA insert to remain flexible."

Accordingly, the Applicant respectfully submits that independent Claims 11 and 28 are patentable over Hodgson because Hodgson does not disclose every element as set forth in those claims. The Applicant further submits that Claims 11-14, 16-18, and 22-23 are patentable over Hodgson because they depend from Claim 11 and define additional patentable subject matter. Withdrawal of the 35 U.S.C. §102 rejection is respectfully requested.

Claim Rejections – 35 U.S.C. §103

Claims 20-21 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Hodgson in view of Nagaki. However, the Applicant respectfully submits that hypothetically combining Nagaki with Hodgson would still fail to teach or suggest the subject matter of Claims 20-21.

To establish a *prima facie* case of obviousness, all of the claim limitations must be taught or suggested by the prior art.

Claims 20 and 21 depend from Claim 11. As discussed above, Hodgson fails to teach or suggest using a DNA compaction agent and "wherein said DNA compaction agent is present at a concentration sufficient to allow the DNA insert to remain flexible," as recited in Claim 11. The rejection turns to Nagaki to solve this problem. However, Nagaki fails to cure the deficiency of Hodgson. Nagaki generally describes ligation of a linearized pUC119 DNA in the presence of HMG. Nagaki does not, however, teach or suggest a DNA compaction agent present at a concentration sufficient to allow the DNA insert to remain flexible, because **there is simply no DNA insert in Nagaki's reaction**. Accordingly, the Applicant respectfully submits that Hodgson and Nagaki, taken individually or in combination, do not render Claim 11 obvious because they do not teach or suggest all the claim limitations. Therefore, Hodgson and Nagaki cannot support *prima facie* obviousness.

Furthermore, neither Hodgson nor Nagaki discloses that a DNA compaction agent is present at a concentration sufficient to allow the DNA insert to remain flexible. Consequently, the unexpectedly superior effects of Claims 20-21 render them particularly well suited for the preparation of circularized recombinant nucleic acids. One skilled in the art would not be able to produce the subject matter claimed in independent Claim 11 based on Hodgson and Nagaki without undue experimentation. Thus, it is not obvious to one skilled in the art to derive the subject matter of Claims 20-21 from the prior art of record. Withdrawal of the 35 U.S.C. §103 rejection is respectfully requested.

All of the stated grounds of rejection have been properly traversed, accommodated, or rendered moot. The Applicant therefore respectfully requests that the Examiner reconsider all presently outstanding rejections and that they be withdrawn. It is believed that a full and complete response has been made to the outstanding Office Action and, as such, the Application is in condition for allowance, which is respectfully requested.

Respectfully submitted,

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DNA Binding of the *Bordetella pertussis* H1 Homolog Alters In Vitro DNA Flexibility

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BpH1, the *Bordetella pertussis* H1 homolog, interacts with chromosomal DNA. With DNase I protection assays, we demonstrate in this study that BpH1 binds DNA in a nonspecific manner and that it may cover DNA fragments from end to end. Although the binding was shown to be nonspecific, preferential binding sites and sites resistant to BpH1 binding were identified within and upstream of the pertussis toxin promoter sequence. In the presence of DNA ligase, BpH1 favored the formation of multimeric DNA fragments of various sizes and prevented ring closures, suggesting a diminished flexibility of the DNA fragments and thus indicating that BpH1 acts as a macromolecular crowding agent.

Many bacteria have genes that code for small basic and relatively abundant proteins, called histone-like proteins, with no significant amino acid sequence similarity to eukaryotic histone proteins. These proteins are associated with either the bacterial chromosome or bacteriophage DNA. The histone-like proteins possess the capability to bind DNA in a nonspecific manner and are able to compact chromosomal DNA many thousand fold (7, 14, 21). In addition, these proteins can regulate transcription of a specific gene by influencing the local promoter DNA supercoiling (6, 12, 13, 16, 24).

We have recently shown that *Bordetella pertussis* directs the synthesis of a small basic and abundant protein, BpH1, that has a high level of amino acid similarity (≈54% identity) to eukaryotic histone H1 (18). Another similar protein, Hc1, has been described for *Chlamydia trachomatis* (9). In both cases, it has been demonstrated that the proteins bind DNA nonspecifically and that they constrain supercoiled structures (2, 18). Furthermore, overexpression of Hc1 in *Escherichia coli* induces condensation of chromosomal DNA (3).

Here, we show that BpH1 binds DNA nonspecifically and that it is able to stimulate intermolecular ligation of linear DNA fragments, very likely by altering DNA flexibility.

Footprinting studies of BpH1 with *Bordetella* DNA. To investigate whether BpH1 has preferential DNA binding sites, we carried out DNase I footprinting experiments with *B. pertussis* promoter and coding regions. DNA probes were from the *bvg* regulatory locus and from the pertussis toxin operon. In *B. pertussis*, the *bvg* locus coordinately regulates expression of virulence factors, while the pertussis toxin is its major virulence factor (1, 4, 8, 10).

The results of a DNase I protection analysis of BpH1 bound to *bvg* promoter fragments are shown in Fig. 1. As expected, at high concentrations of BpH1, the probes were completely insensitive to DNase I digestion (Fig. 1A and B, lanes 3). With addition of competitor DNA, the two probes were not bound by BpH1, giving rise to a pattern of DNase I digestion indistinguishable from that of the naked DNAs (Fig. 1A and B, lanes 4 to 6). DNase I protection experiments with BpH1 bound to fragments from the coding regions of the filamentous hemagglutinin (5) and pertussis toxin (8) genes gave similar results (data not shown). We conclude that binding of BpH1 to these DNA fragments is not specific.

The results of footprinting experiments carried out with DNA strands within and upstream from the P_{TOX} promoter region of the pertussis toxin operon are shown in Fig. 2. Surprisingly, a 12-base region resistant to BpH1 binding could be clearly detected (Fig. 2A, solid bar). This region maps from position −37 to position −26 (5′-ACCCCCCTGCCA) with respect to the transcription start point of the P_{TOX} promoter (8). Additional sites resistant to BpH1 binding could be detected on the same DNA probe. Among these BpH1-resistant sites, five sites (used also as reference bands) map to positions +12, −1, −7, −8, and −59 with respect to the transcription start point. Interestingly, in the presence (Fig. 2A, lane 4) or absence (lane 3) of 50 ng of competitor DNA, the footprint indicates altered DNase I sensitivity in a region 11 bp long mapping from position +1 to position +11 (Fig. 2, shaded bar). DNA regions upstream of positions −10 and −60 show a similar altered DNase I sensitivity. As the amounts of competitor DNA were increased to 100 and 200 ng, these sites were progressively not evident, suggesting that, although not specific, the sites could represent preferential binding sites of BpH1.

Results obtained by using the same DNA probe labeled at the opposite extremity are shown in Fig. 2B. This probe allowed the analysis of BpH1 binding upstream of the P_{TOX} promoter region. Two sites located 14 bp apart and resistant to BpH1 binding could be detected (Fig. 2B, solid triangles). These sites delimit DNA regions that gave altered sensitivity to DNase I. The binding of BpH1 to this region was progressively inhibited by the presence of competitor DNA. In addition, a slight enhancement of DNase I sensitivity could be detected at position −292 (marked by an arrow).

These results indicate that the P_{TOX} DNA region contains preferential BpH1-binding sites and BpH1-resistant sites. Interestingly, one BpH1-binding site maps in the proximity of the transcription start point, a relatively T-rich region, whereas a BpH1-resistant binding site consists of 13 bp centered around position −30 from the transcription start site.

BpH1 stimulates ligation of linear DNA fragments. It is well established that changes in the local structure of a promoter DNA sequence, mediated either by environmental changes or

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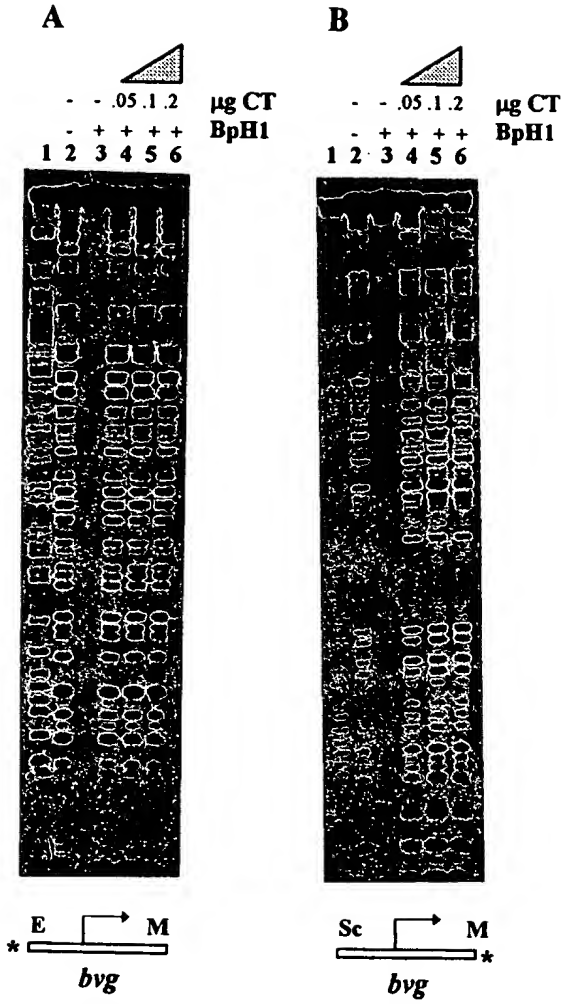

FIG. 1. Footprint analyses of BpH1 bound to the upper (A) or lower (B) DNA strand of the *bvg* promoters. The probes are schematically represented below the figures (drawings are not to scale), and their sites of ^{32}P end labeling are indicated by asterisks. Probes (126-bp *Eco*RI-*Mlu*I [A] and 141-bp *Sac*II-*Mlu*I [B]) were purified from plasmids pVIR/EP and pPROM-67 (19, 20), respectively. E, *Eco*RI; M, *Mlu*I; Sc, *Sac*II; CT, calf thymus competitor DNA. Arrows indicate the direction of transcription. In lanes 1, a G+A sequence reaction product (17) served as the size marker. BpH1 was purified as previously described (18). Complexes were formed in 50 µl of footprint buffer (10 mM Tris-Cl [pH 8], 10 mM NaCl, 0.5 mM $MgCl_2$, 0.25 mM $CaCl_2$, 1 mM dithiothreitol, 20 µg of bovine serum albumin per ml, 10% glycerol) containing ca. 10 fmol of DNA and 100 ng of BpH1 for 15 min at room temperature. One microliter of DNase I (0.2 U/µl) in footprint buffer with 5 mM $CaCl_2$ was added, and incubation was continued for 30 s at room temperature. DNase I digestion was stopped by addition of 140 µl of stop solution (192 mM sodium acetate, 32 mM EDTA, 0.14% sodium dodecyl sulfate, 64 µg of yeast RNA per ml). Samples were extracted with phenol-chloroform, precipitated with ethanol, resuspended in 5 µl of loading buffer, denatured at 95°C for 5 min, loaded onto an 8 or 10% polyacrylamide sequencing gel, and autoradiographed.

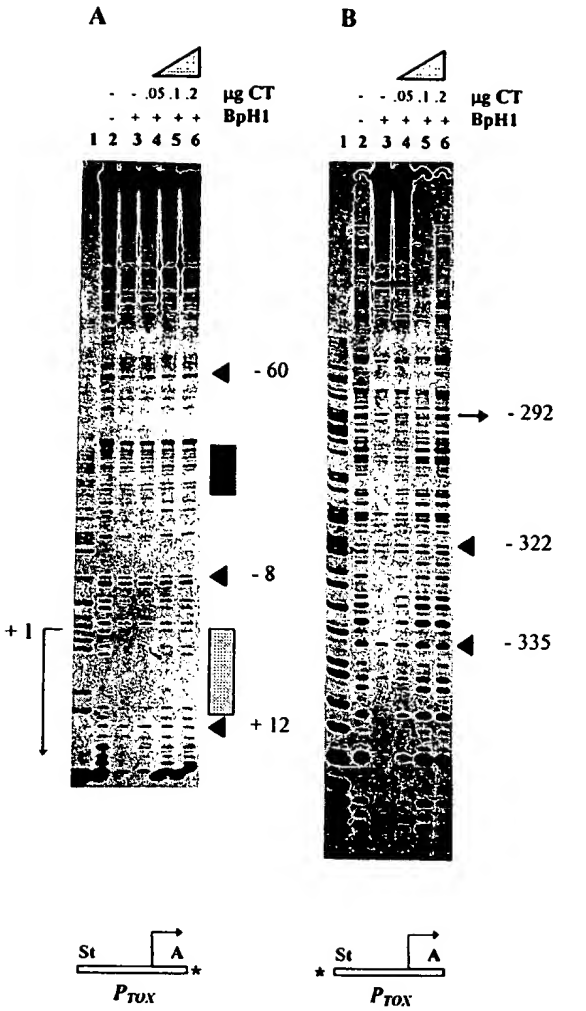

FIG. 2. Footprinting analyses of the BpH1 protein bound to the P_{TOX} promoter (A) or the P_{TOX} upstream region (B). The probes are schematically represented below the figures (not to scale), and their sites of ^{32}P end labeling are indicated by asterisks. These probes (316-bp *Stu*I-*Ava*II) were prepared from plasmid pT1/EP (19, 20). St, *Stu*I; A, *Ava*II. A main domain of the BpH1 preferential binding site is indicated by a shaded bar. Solid symbols indicate sites of noninteraction of BpH1 with DNA. The amounts of calf thymus (CT) competitor DNA are indicated. Lanes 1, a G+A sequence reaction product. Experimental conditions were as described in the legend to Fig. 1.

by histone-like proteins (6, 12, 13, 16, 24, 25), may influence expression of a specific set of genes. To assess whether the peculiarity of binding of BpH1 to the P_{TOX} promoter region could induce bending or structural changes in the DNA, we have carried out ligation of labeled small DNA fragments from this region in the absence and presence of BpH1.

Ligation of a 77-bp DNA fragment that normally cannot circularize (15, 22) gave rise to a barely detectable band migrating at a position of 154 bp (Fig. 3A, lane 4). As expected, this band was sensitive to exonuclease III digestion (lane 5). Interestingly, by addition of increasing amounts of BpH1, the intensity of this band increased (lanes 6 and 8), and two other bands with sizes of 231 and 308 bp appeared. Surprisingly, all of these DNA bands were sensitive to exonuclease III digestion (lanes 5, 7, and 9); therefore, they represented multimerization of the linear DNA fragment. Similar results were obtained with a 154-bp-long DNA probe (Fig. 3B). These data show that BpH1 could stimulate ligation of linear DNA fragments and did not induce circularization through bending of the DNA molecules. However, we cannot rule out the possibility that BpH1 bound to DNA could induce other types of structural DNA modifications.

To investigate whether this mechanism is restricted to the P_{TOX} promoter region or is related to DNA in general, we have ligated plasmid DNA in the presence of increasing

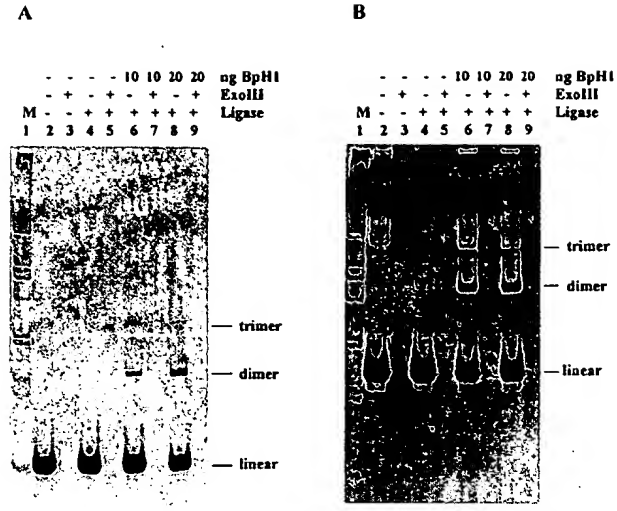

FIG. 3. BpH1-mediated ligation of short DNA fragments. (A) The ^{32}P-end-labeled 77-bp DNA fragment was ligated in the absence (lanes 4 and 5) or presence (lanes 6 to 9) of the indicated amounts of BpH1. The protein-DNA binding was carried out for 30 min at room temperature in 100 μl of 10 mM Tris-Cl (pH 7.5)–5 mM $HgSO_4$–1 mM dithiothreitol–0.1 μg of bovine serum albumin per ml. Samples were incubated with T4 DNA ligase (5 U) for 15 h at room temperature, extracted with phenol-chloroform–2 M NaCl, ethanol precipitated, electrophoresed on native 7% polyacrylamide gel in Tris-borate-EDTA buffer, dried, and exposed to X-ray film for autoradiography. Samples treated with exonuclease III (ExoIII) are indicated. The probe was obtained by PCR with plasmid pT1/ES (19, 20) by using the oligonucleotides $^{-181}$Z3 (5′-CAACCGCCAACGCGCAT) and $^{-105}$Z2 (5′-TGGATTGCAGTAGCGGG). The superscript numbers refer to the map positions with respect to the P_{TOX} transcriptional start point (8). Lane 1 (M) contained a ^{32}P-labeled 1-kb ladder (Gibco-BRL) as the size marker. (B) The ^{32}P-end-labeled 145-bp DNA fragment, obtained by PCR with the primers $^{-70}$T-70 (5′-GCGCGACTTTGCGC CGAAG) and $^{-214}$Z1 (5′-CGTCCGGCCGGCACCAT), was used as a probe. It is worth noting that this DNA probe migrated with an apparent molecular size slightly larger than the expected size.

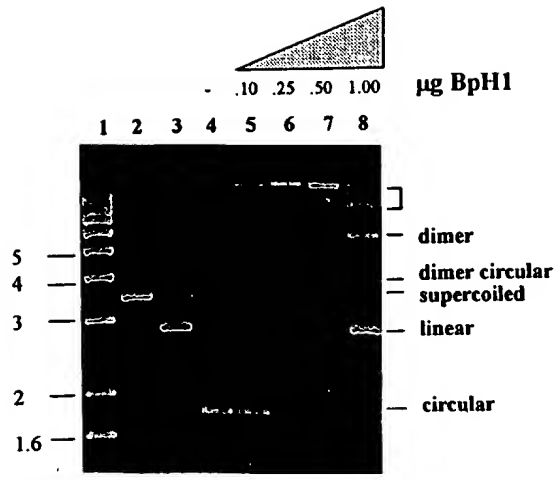

FIG. 4. BpH1-mediated ligation of 1 μg of *Eco*RI-linearized pGem4 plasmid DNA (Promega). Ligation was performed in the presence of the indicated amounts of BpH1. Supercoiled plasmid (lane 2), *Eco*RI-linearized plasmid (lane 3), and a 1-kb ladder (lane 1) served as size markers. Numbers on the left side are in kilobases. The protein-DNA binding was carried out as described in the legend to Fig. 3, and samples were electrophoresed on a 1% agarose gel containing 20 μg of chloroquine per ml in Tris-borate-EDTA buffer for 15 h at 5 V/cm. Gels were washed in water for 4 h and stained with 1 μg of ethidium bromide per ml.

amounts of BpH1. To resolve possible BpH1-induced supercoils, we fractionated the ligated bands on chloroquine-containing agarose gels. In the absence of BpH1, most of the linear DNA was converted into monomer circular DNA (Fig. 4, lane 4). In the same lane, we could see a band migrating to a position corresponding to that of the most supercoiled plasmid (marked supercoiled) and a band migrating to a position slightly higher, which is likely to correspond to the position of the dimer circular plasmid. A DNA band with a molecular size greater than 12 kb was also detectable. With addition of increasing amounts of BpH1 to the ligation mix, the pattern of bands of ligated DNAs progressively changed. Up to a concentration of 500 ng of BpH1 (Fig. 4, lane 7), the monomer circular DNA disappeared, as did the supercoiled and the dimer circular bands, whereas the amount of the highest-molecular-weight band increased. Surprisingly, by using 1 μg of BpH1, most of the linear DNA remained unligated (Fig. 4, lane 8), while dimers of linear DNA and higher-molecular-weight bands (indicated by a bracket) were detected.

At a high concentration of BpH1, the plasmid DNA is likely to be entirely covered by the protein from one end to another, thus favoring the formation of linear DNA catenanes by ligase. BpH1 renders DNA fragments extremely rigid and therefore reduces the degree of DNA flexibility. The effect of a BpH1-mediated ligation is similar to that exerted by the addition of polyethylene glycol to a ligase reaction mixture or similar to that exerted by the addition of histone H1 (23), suggesting that BpH1 acts as a macromolecular crowding agent. On the contrary, a large excess of BpH1 in the mix inhibits ligation of DNA fragments, possibly by even occluding the extremities of the DNA.

Whether BpH1 could also regulate transcription of specific genes by altering local DNA structures remains to be investigated. However, preliminary data indicate that, as in the case of Hc1 of *C. trachomatis* (2), overexpression of BpH1 under the control of an inducible promoter (11) in *E. coli* interfered with bacterial multiplication. The BpH1-expressing cells grew for nearly 100 min after induction and then entered the stationary phase of growth. Cells not expressing BpH1 grew for at least an additional 200 min. Although not investigated, this change in bacterial multiplication is likely to be accompanied by changes in DNA replication and other biological functions (2).

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DNA Bending Induced by the Archaebacterial Histone-like Protein MC1

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The conformational changes induced by the binding of the histone-like protein MC1 to DNA duplexes have been analyzed by dark-field electron microscopy and polyacrylamide gel electrophoresis. Visualisation of the DNA molecules by electron microscopy reveals that the binding of MC1 induces sharp kinks. Linear DNA duplexes (176 bp) which contained a preferential site located at the center were used for quantitative analysis. Measurements of the angle at the center of all duplexes, at a fixed DNA concentration, as a function of the MC1 concentration, were very well fitted by a simple model of an isotropic flexible junction and an equilibrium between the two conformations of DNA with bound or unbound MC1. This model amounts to double-folded Gaussian distributions and yields an equilibrium deflection angle of $\theta_0 = 116°$ for the DNA with bound MC1. It allowed measurements of the fraction of DNA with bound MC1 to be taken as a function of MC1 concentrations and yields an equilibrium dissociation constant of $K_d = 100$ nM. It shows that the flexibility of DNA is reduced by the binding of MC1 and the formation of a kink. The equilibrium dissociation constant value was corroborated by gel electrophoresis. Control of the model by the computation of the reduced χ^2 shows that the measurements are consistent and that electron microscopy can be used to quantify precisely the DNA deformations induced by the binding of a protein to a preferential site.

Keywords: DNA bending; DNA flexibility; architectural protein; MC1; electron microscopy

Introduction

Numerous chromosomal proteins have been isolated from prokaryotic nucleoids. These abundant, small and usually basic proteins are generally referred to as histone-like proteins. They compact DNA *in vitro*, and can play essential roles in different cellular processes (Drlica & Rouvière-Yaniv, 1987; Gualerzi *et al.*, 1986; Schmid, 1990; Pettijohn, 1990). In *Escherichia coli*, histone-like proteins seem indispensable and the complete absence of the HU protein is deleterious to the cells (Wada *et al.*, 1988; Boubrik & Rouvière-Yaniv, 1995). Whereas the proteins HU, IHF, and H-NS are highly conserved in eubacteria, different proteins have been found in archaea. In most cases, the primary sequences of the archaeal structural proteins are more similar within one species than with other archaea, and HTa, HMf, and MC1 have no sequence homology (Grayling *et al.*, 1996). In some archaea, nucleosome-like structures have been visualized by electron microscopy (Sandman *et al.*, 1990). Furthermore, archaeal nucleosomes have been isolated from *Methanobacterium thermoautotrophicum* and *Methanothermus fervidus* (Pereira *et al.*, 1997). Such structures do not seem to exist in the *Methanosarcina* species that contain the protein MC1 (Imbert *et al.*, 1988).

The protein MC1 has been isolated from *Methanosarcina* sp. CHTI 55 and sequenced (Laine *et al.*, 1986; Chartier *et al.*, 1988; 1989). It is a 93 amino acid residue polypeptide and a basic protein with a net charge of +12. It exhibits a marked hydrophilic character provided by a large number of basic and acidic residues and no large hydrophobic domain. The N-terminal region of the protein (residues 1-58) contains a very conserved sequence, and four prolyl residues which are exactly conserved in the C-terminal region (residues 59-93) of

Abbreviations used: EM, electron microscopy; *r*, ratio of protein to DNA concentrations.

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proteins from several different species of *Methanosarcina* (Laine *et al.*, 1990).

MC1 is an abundant protein *in vivo* with an estimated concentration ratio of one molecule per 170 bp (Imbert *et al.*, 1988). MC1 preferentially binds double-stranded DNA, and protects DNA against thermal denaturation (Chartier *et al.*, 1988). The protein binds to DNA as a monomer and is able to cover linear DNA fragments non-cooperatively with a site size of about 11 bp at a relatively high ionic strength and a high protein/DNA ratio (Culard *et al.*, 1993).

Cyclisation experiments of short DNA fragments of 110 bp (Laine *et al.*, 1991) with bacteriophage T4 DNA ligase suggest that MC1 induces large bends in DNA. With fragments of 203 bp, MC1 induces the formation of topoisomers (Laine *et al.*, 1991). Although MC1, like all packaging proteins, is not expected to exhibit absolute specificity for one particular sequence, a preferential binding sequence has been identified in an open reading frame of unknown function (Teyssier *et al.*, 1994). DNA supercoiling favors MC1 binding, even without a preferential binding site (Teyssier *et al.*, 1996). MC1 forms at least two types of binding complexes with relaxed DNA minicircles, these complexes corresponding to a stoichiometry of one or two MC1 molecules per DNA minicircle, where each bound protein induces a sharp bend of the DNA, as observed by electron microscopy (EM) and atomic force microscopy (Toulmé *et al.*, 1995; Larquet *et al.*, 1996).

DNA compaction and many DNA transactions such as replication, transcription, and recombination, involve DNA deformation by proteins into specific conformations. Specific sequences, local bendability of DNA or particular DNA conformations can be the target of such architectural DNA binding proteins. Numerous chromosomal binding proteins such as HU, IHF, TF1 and HMG1/2 illustrate the importance of DNA conformation for protein binding and show that specific conformational changes are associated with the DNA-protein interaction to major or minor grooves (Bianchi *et al.*, 1989; Chow *et al.*, 1994; Locker *et al.*, 1995; Grove *et al.*, 1996; Bewley *et al.*, 1998). Classical methods used to investigate and to analyze DNA bending induced by protein, such as circular permutation or phasing analysis, require the existence of specific binding sites. This requirement is even more stringent for NMR and crystallographic structural analyses.

The conformational change induced by non-specific binding of histone-like proteins such as MC1 is experimentally difficult to analyze. *A priori* the binding of the protein induces a bend with a geometry which does not depend on the effective position in the DNA fragment. However, different positions of the same bend in the DNA fragment induce different electrophoretic mobility of the DNA-protein complex. Reduction of migration is maximal when the bend is located at the center and minimal when it is located near the end (Wu & Crothers, 1984). As a result, observations by gel electrophoresis are difficult to interpret because they correspond to the global outcome of the migrations of a large number of different configurations for the binding protein-DNA complexes. Furthermore, it is difficult to distinguish, by gel electrophoresis, local bending from local flexibility, because this method measures a macroscopic variable, the retardation, as the global outcome of both phenomena. In the case of MC1, these results are particularly difficult to interpret due to the moderate specificity of the MC1 binding (Teyssier *et al.*, 1994).

With electron microscopy techniques, the detailed contours of individual molecules can be recorded and the deformations induced by ligands can be analyzed along the DNA. With the analysis techniques developed in the accompanying paper (Cognet *et al.*, 1998), we can characterize quantitatively the deformations induced by the binding of MC1 to DNA for a large number of molecules. Here, after showing the difficulty in characterizing the randomly located bend in a linear DNA fragment of 203 base-pairs which does not exhibit a specific MC1 binding site, we have demonstrated how we have applied these methods for the characterization of the protein binding and of DNA bending at a preferential binding site located at the center of a 176 base-pair fragment. The latter study requires the careful control of the concentration ranges of the MC1 protein and of the DNA so that most of the binding occurs at the preferential binding site and not at non-specific sites on the remaining parts of the DNA fragments. As a result, binding has also been studied by gel retardation, and the equilibrium binding constant for this reaction has been measured by EM and gel methods to characterize the bending angle in these experiments.

Results

DNA kinks upon MC1 binding: non-specific and preferential binding sites

Electron micrographs of the MC1-DNA complexes demonstrate that MC1 induces a drastic conformational change observed as one or several important kinks in the DNA molecules (Figures 1b and 2b). Such kinks are infrequent in the absence of MC1, as shown in the control experiments (Figures 1a and 2a). Although MC1 (11 kDa) is too small to be directly observed by the EM procedure used here (Toulmé *et al.*, 1995), its binding site positions can be inferred from the mere observations of the large induced kinks.

These kinks are located at random positions along the 203 bp DNA molecules shown in Figure 1b and at the center of the DNA fragments shown in Figure 2b. In the former case, the 203 bp DNA fragment does not contain any preferential binding site, in agreement with the observed random kink positions (Figure 1b), whereas in the lat-

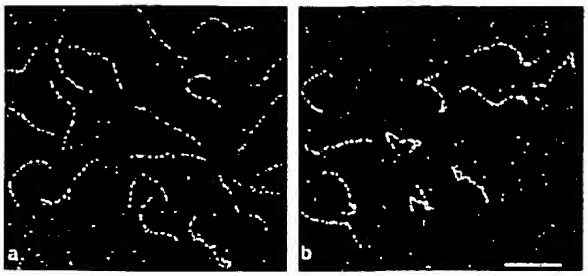

Figure 1. Electron micrographs of MC1-DNA complexes: a, 203 bp control DNA fragment; b, MC1 bound to 203 bp fragment; the MC1:DNA ratio was 7:1. The final DNA concentrations were 7.5 μM and 8.6 μM, respectively. The bar represents 50 nm.

ter, we have chosen a 176 bp fragment containing a preferential binding site located at its center (Figure 2b).

To characterize the kink angle values on the first fragment, angles were measured at each deformation observed along the DNA contour lines. The population distribution of the collected experimental values in Figure 3 shows a broad spectrum range. This is probably due to the difficulty of collecting statistics for kink angles. As shown above, these kinks result from the binding of MC1, but can also result from normal deformations by thermal agitation in the absence of the protein. Furthermore, some deformations can result from the close

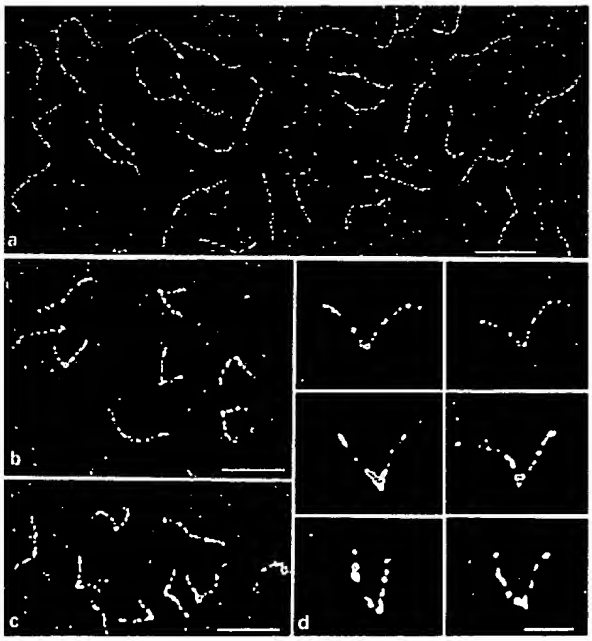

Figure 2. Electron micrographs of MC1-DNA complexes: a, 176 bp control DNA fragment; b, interaction of MC1 with 176 bp DNA fragments containing a preferential binding site for MC1 located at their center. The final DNA concentrations were 7.5 μM and 8.6 μM, respectively. The bars represent 50 nm (a,b,c) and 20 nm (d).

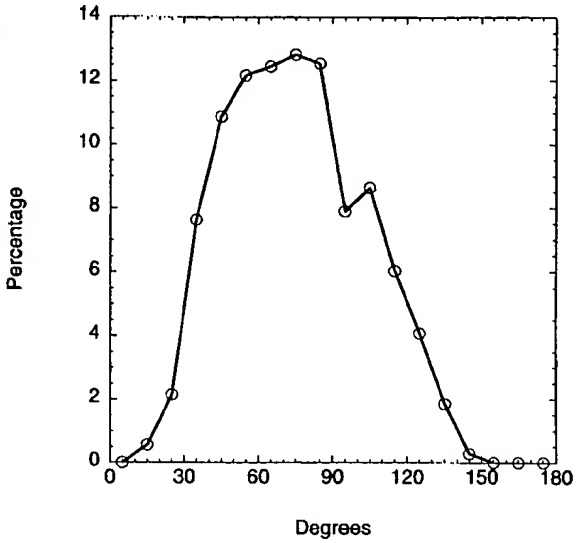

Figure 3. Plot of the distribution of 1076 kink angles measured on linear 203 bp DNA fragments in the presence of MC1 (the MC1:DNA ratio was 7:1).

binding of several MC1 molecules to adjacent DNA locations.

Three folded Gaussian distributions, or more, would be required to fit these data. Therefore no clear kink angle can be measured from data collected with non-specific binding. However, the deformations observed in Figure 2b with the DNA containing the preferential binding site are well localized at the center, and the molecules display practically no other major deformation elsewhere along the DNA. This suggests a single binding site for MC1 on the DNA fragment. The resulting DNA deformation is characterized and quantified below.

Measurement of DNA bending induced by MC1 at the preferential binding site as a function of MC1 concentration

We have recorded the contours of the 176 bp DNA fragments that contain a preferential binding site at their centers. Two situations must be considered: at high DNA and protein concentrations, binding of MC1 may occur everywhere on the DNA molecule as well as at the preferential binding site. At low concentrations, binding, if any, occurs mostly at the preferential binding site. The concentration limit between these two cases is given by the equilibrium dissociation constant, K_d, for the preferential binding site. This is why the following experiments were performed with a fixed DNA concentration and variable MC1 to DNA concentration ratio, r.

In the accompanying paper (Cognet *et al.*, 1998), we have shown that angle measurement requires that the length of the segments should be more than 20-30 bp, in order to yield reliable measure-

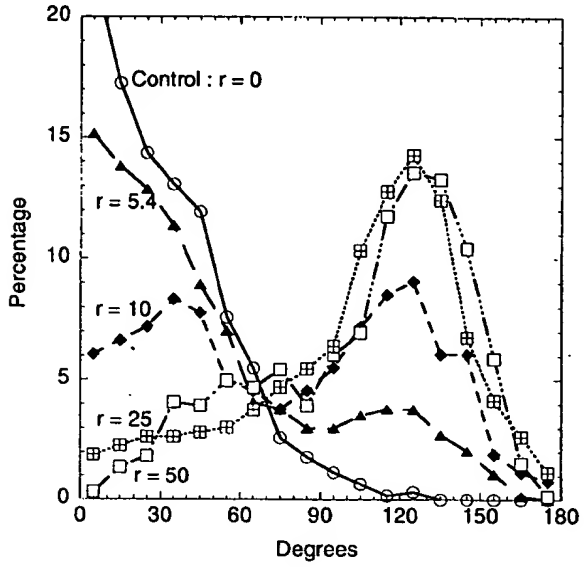

Figure 4. Plot of the percentage of the observed population distribution of kink angles values, in ten degree intervals, measured at the center of the linear 176 bp DNA fragments containing a preferential binding site for the protein MC1, in the presence of MC1 at a fixed DNA concentration (8.6 nM) and at a variable ratio, $r = [MC1]/[DNA]$. Distributions with $r = 0$ (○) and $r = 5.4$ (▲) correspond to observations obtained directly on the EM grid after mixing the DNA and the protein, whereas distributions with $r = 10$ (◆), 25 (⊞), and 50 (⊡) were collected on the EM grids after purification of the complexes by chromatography.

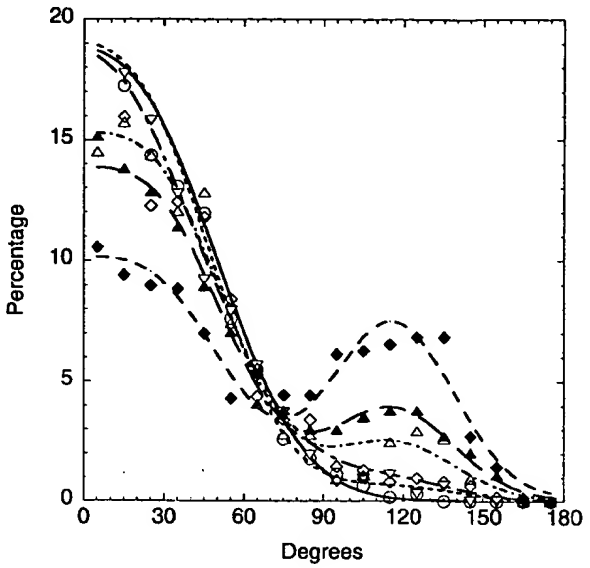

Figure 5. Scattered plots with their fitted curves of the percentage of the observed population distribution of kink angle values, in ten degree intervals, measured on the linear 176 bp DNA fragments, as in the legend to Figure 2, at variable ratios, $r = [MC1]/[DNA] = 0$ (○), 0.53 (▽), 1.1 (◇), 2.7 (△), 5.4 (▲), and 10.8 (◆). These distributions correspond to observations obtained directly on the EM grids after mixing the DNA and the protein. The curve fits are a sum of two folded Gaussian distributions with the parameters given in Table 1A.

ments. In the present analysis, the long segments may be deformed by the non-specific binding of MC1. In order to limit the complexity of the contour analyses, angles have been measured with sides fixed at the intermediate length of 50 bp.

Measurements were performed on a large number of molecules (>500) and a sample of typical distributions of kink angles measured at the center of the molecules, at different ratios, is shown in Figure 4. The first distribution in Figure 4 ($r = 0$) is the control experiment without MC1 and corresponds to "straight" DNA. As r increases, the population distribution is shifted towards approximately 120° with an isobestic point. This demonstrates the existence of two populations of DNA molecules without and with MC1 bound at the center of the DNA fragments.

The results of two series of experiments A and B are shown, respectively, in Figures 5 and 6 and are summarized in Table 1. At the low ratios of the A series ($r = 0$; 0.53; 1.1; 2.7; 5.4; 10.8), single binding can be characterized by a direct observation on the EM grids of the incubation complex, without any further purification. At the high ratios of the B series ($r = 5$; 10; 25; 50), optimal conditions for visualization of protein-DNA complexes required their purification by gel filtration to remove unbound protein and to reduce non-specific binding.

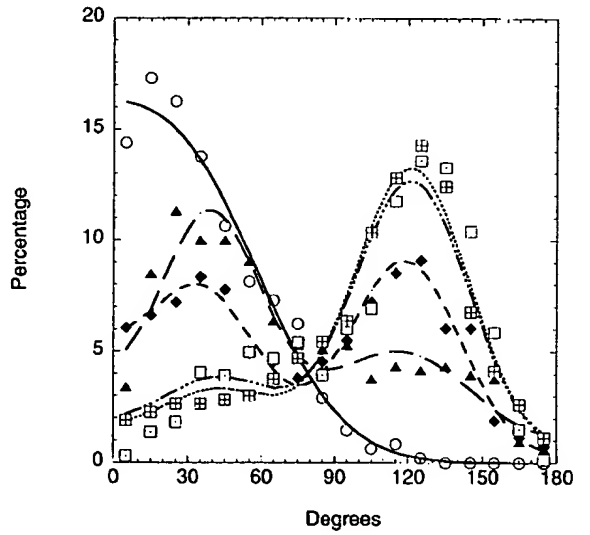

Figure 6. Scattered plots with their fitted curves of the percentage of the observed population distribution of kink angle values, in ten degree intervals, measured on the linear 176 bp DNA fragments, as in the legend to Figure 2, at variable ratios, $r = [MC1]/[DNA] = 0$ (○), 5 (▲), 10 (◆), 25 (⊞) and 50 (⊡). These distributions were collected on the EM grids after purification by chromatography. The curve fits are a sum of two folded Gaussian distributions with the parameters given in Table 1B.

Table 1. The average angle kink values and their associated flexibilities as a function of the concentrations ratio, r, of MC1 to DNA fragments

Molar ratio r = [Total MC1]/[DNA]	n	aa	θ_a(°)	σ_a(°)	θ_b(°)	σ_b(°)	χ^2 @ 0.95 (degrees of freedom)	χ^2 of data
A. *Without gel filtration*								
0	608	0 (np)	25	30	np	np	21.0 (12)	11.1
0.53	620	0.04	24	28	116 (np)	23 (np)	21.0 (12)	11.4
1.1	622	0.05	14	38	116 (np)	23 (np)	21.0 (12)	20.4
2.7	648	0.14	27	28	116	23	64.0 (47)	59.6
5.4	739	0.23	27	28	116	23	64.0 (47)	59.6
10.8	702	0.43	27	28	116	23	64.0 (47)	59.6
B. *After gel filtration*								
0	480	np	29	33	np	np	21.0 (12)	14.4
5	531	0.40	38	22	116	31	21.0 (12)	14.4
10	528	0.52	34	24	118	23	21.0 (12)	7.2
25	663	0.75	42	26	121	23	79 (60)	115
50	532	0.78	42	26	121	23	79 (60)	115

In these experiments, the concentration of DNA was held fixed (8.61 nM): n is the number of molecules sampled: $(1-aa)$ is the fraction of the molecules that belong to the first Gaussian distribution (θ_a, σ_a) that corresponds to unbound DNA: aa is that of the second Gaussian distribution (θ_b, σ_b) that corresponds to the DNA fragments with bound MC1. Best-reduced χ^2 values obtained for the fit with these Gaussian distributions are given in the last column and should be compared to the numbers of the last but one column that indicate the χ^2 at 0.95 significance; the numbers of degrees of freedom used in the fit are in parentheses. Experiments with molar ratios r= 2.7, 5.4, and 10.8 were fitted together. As the distributions that correspond to molar ratios $r = 25$ and 50 did not fit well due to non-specific and multiple bindings, they were fitted together with those of $r = 5$ and 10 to obtain estimates of aa as a function of the molar ratio. Experiments of series A were visualized directly whereas those of series B at high r ratios were visualized after gel filtration.

np, not a parameter.

The control experiments in the absence of MC1 are well fitted by a single folded Gaussian with parameters θ_a, the average angle value, and σ_a, the standard deviation, as shown in Figures 5 and 6, and by the values of the reduced χ^2 given in Table 1. At intermediate concentrations, (A ratios $r = 2.7$, 5.4, 10.8; B ratios $r = 5$, 10), the distributions are well fitted by the sum of two folded Gaussians, where $(1 - aa)$ is the weight of the first distribution and aa the weight of the second. The first folded Gaussian is described by the parameters θ_a and σ_a, and the second Gaussian by θ_b, and σ_b. Control DNA, which is characterized by $\theta_a = 25\text{-}29°$ and $\sigma_a = 28\text{-}33°$, is deformed by MC1 into kinked DNA characterized by $\theta_b = 116°$ and $\sigma_b = 23°$. As the latter values of kinked DNA are well defined, angle distributions obtained at the low concentration A ratios $r = 0.53$ and 1.1 were fitted to the sum of two folded Gaussians where the second Gaussian had fixed parameters (116°, 23°). These distributions and the results collected in Table 1 demonstrate the progressive formation of the MC1-DNA complex with increasing MC1 concentrations.

At high MC1/DNA ratios, chromatography purification was required for good visualization of the complexes by EM. For this reason, experiments at high MC1/DNA B ratios ($r = 5$; 10; 25; 50) are different but closely related to experiments of the A series, as shown by the agreement obtained for θ_a, σ_a, θ_b and σ_b in the region where the MC1/DNA ratios overlap, $r = 5$ and 10. When the ratio r increases from 0 to 50, close analysis of the distribution curves of the B series shows that the mean value of "straight" DNA, θ_a, tends to increase from 29° to 42° while the distributions of the data broaden, as shown in Figure 5 (note that global fits and not individual fits are shown at high ratios in Table 1). This indicates the progressive contribution of non-specific binding as reflected by the poor score χ^2 at the large concentration ratios ($r = 25$, 50).

Equilibrium dissociation constant determined from gel electrophoresis and from electron microscopy data

The same sample solutions, mixed at the same concentrations and the same salt conditions, as for the EM experiments were loaded on a gel for electrophoresis. The results are shown in Figure 7. Up to a ratio of MC1/DNA of 10, we observe a major band that corresponds to a complex including one MC1 molecule per DNA fragment. At higher MC1 concentrations, additional bands are observed. They correspond to increasing numbers of MC1 molecules binding non-specifically to the fragment. These results show that the protein concentrations used in the EM experiments are in the appropriate range to study the binding of one MC1 molecule to its preferential site, but they also confirm that the binding is only preferential. The fraction of bound DNA, measured by scanning the gel, is shown in Figure 8 as a function of free [MC1]. Fitting these data to a single binding site equilibrium curve, $Y = [\text{MC1}]/(K_{\text{dgel}} + [\text{MC1}])$, gives an estimated value for the equilibrium dissociation constant $K_{\text{dgel}} = 40$ nM. As the total DNA concentration is small with respect to K_d, the fit is not sensitive to exact MC1 concentrations: if total MC1 concen-

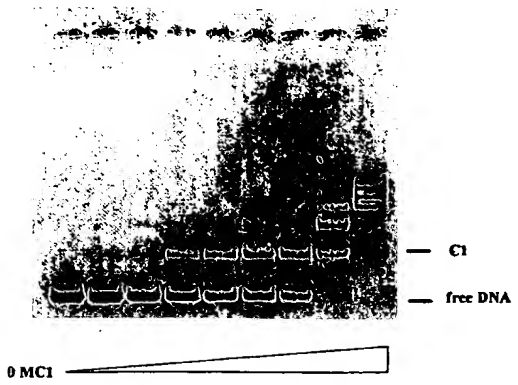

Figure 7. Titration of the linear 176 bp DNA fragment with MC1 protein. Varying amounts of MC1 were mixed with ^{32}P-labeled 176 bp DNA fragment (8.6 nM) in 10 mM Tris-HCl (pH 7.5) 50 mM NaCl. The different complexes were resolved on a low-ionic gel (5% polyacrylamide). Gels were dried and autoradiographed with CGR films. The [MC1]/[DNA] ratios are from left to right, $r = 0$; 0.25; 0.5; 1; 2.5; 5; 10; 25; 50.

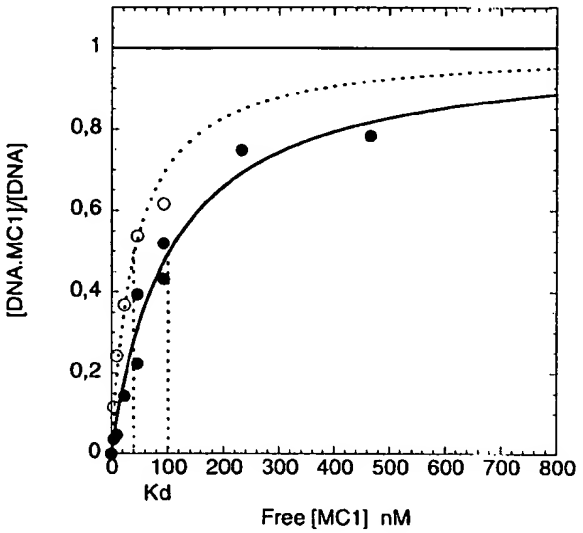

Figure 8. Plots of the saturation functions [DNA·MC1]/[DNA] obtained by gel electrophoresis (open circles and broken lines) and by EM (filled circles and continuous line) as a function of free [MC1] concentrations. The fractions of DNA with bound MC1 were obtained by scanning the gel bands shown in Figure 7. The broken curve is the best fit hyperbola to the gel data with the equilibrium dissociation constant, $K_d = 42$ nM. The fractions of DNA with bound MC1 were obtained by analysis of EM data. They are the values, *aa*, obtained by fitting the distributions of Figures 4 and 5 to a sum of two folded Gaussian distributions as summarized in Table 1A and B. The continuous curve is the best fit hyperbola to the EM data with the equilibrium dissociation constant $K_d = 102$ nM.

trations were used instead of free concentrations, the K_d would be increased by 10%.

The fraction of the DNA molecules with bound MC1, Y, is obtained from the analysis of EM data and can be read from column *aa* of Table 1. It is plotted as a function of free [MC1] as shown in Figure 8. These data fit well to a single binding site equilibrium curve, $Y = [MC1]/(K_{d(EM)} + [MC1])$. It gives the equilibrium dissociation constant $K_{d(EM)} = 102$ nM. As above, if the total MC1 concentrations were used instead of free MC1 concentrations, the K_d would be increased by less than 5%.

Discussion

We have characterized quantitatively the binding of MC1 and its capability of bending DNA. Electron micrographs show large and well-localized kinks in the DNA linear fragments induced by MC1. Large conformational changes upon binding have been illustrated for MC1 binding to DNA minicircles, but the DNA deformation could not be quantified (Toulmé *et al.*, 1995). Electron micrographs show that MC1 bends the DNA fragments, whether or not they contain a preferential binding site. Gathering quantitative information on bends is uphill work in the absence of a preferential site. Firstly, MC1 induces the formation of structures with random bends which are too complex for analysis, and secondly, as MC1 is not directly visible, it is difficult to correlate unambiguously the observed bends to protein binding. For these reasons, one cannot state whether or not the bending of DNA at a non-specific site is identical with that observed at the preferential site. The existence of a preferential binding site located at the center of the DNA fragment has allowed restriction of the angle measurements to within a narrow region of the fragment. This has proved to be essential to perform quantitative measurements and to check the validity of our assumptions.

Detailed measurement of bending at a preferential site

The strict repetition of the method developed in the accompanying paper (Cognet *et al.*, 1998) would have required the detailed analysis of the digitized contours of the DNA molecules to obtain the mean angle value of the induced bend and its local flexibility. To take advantage of the conclusions developed here and to simplify the analysis, the angles were systematically measured at the center of the DNA fragments with adjacent sides of about 50 bp. This 50 bp length is well above the global resolution of the electron micrographs and of the method, as shown by Cognet *et al.* (1998). It is short enough to avoid the small intrinsic deformations observed in these DNA sequences. It is long enough to account for some of the uncertainty due to the non-specific location of binding. Therefore, this is a very practical and rapid method that can be used for a very large number of molecules.

This simplified procedure has allowed us to bypass three major difficulties. Firstly, as discussed below, the binding of MC1 to the central site is only preferential, and MC1 may bind to other sites along the DNA fragment at high protein concentrations. This important feature could introduce some uncertainty about the localization of the bend, and renders the analysis much more complex at high MC1 concentrations or high MC1/DNA ratios. Secondly, the sequences of the 203 bp and 176 bp DNA fragments contained minor intrinsic bends or minor local flexibilities as observed by EM of the control DNA fragments without MC1. Thirdly, such a detailed analysis would be computationally demanding as explained below.

Because MC1 is too small to be visualized by EM, only the conformational change can be correlated with the binding site of the protein. The bend at the center of the DNA fragment depends on the thermal fluctuations and on the effective binding of MC1. Therefore all of the angles of the DNA fragment fully contained in the image frames were included in the analysis, and were measured as a function of MC1 concentration. This increased considerably the number of angle measurements. A group of 500 angle measurements constitute a minimal statistical set to unravel the five parameters associated with naked DNA fragments (θ_a, σ_a), DNA with bound MC1 (θ_b, σ_b), and the fraction, *aa*, of DNA with bound MC1 at a given MC1 concentration. Studies of these parameters as a function of MC1 concentrations have required a number of angle measurements of well over 6000 images of molecules.

The choice of the folded Gaussian model

The Gaussian distribution is particularly simple and well adapted to describe the population distribution of angles at a kink or a bend in double-stranded DNA, as explained in the accompanying paper (Cognet *et al.*, 1998). Since angle values accessible to experimental measurement comprising those between 0 and 180°, the experimental distribution should follow a folded Gaussian distribution (Le Cam *et al.*, 1994). As a consequence, all average angle values and flexibilities can only be obtained by fitting the experimental data to a folded Gaussian distribution. This method has proved to be very useful for analyzing a 139 bp double-stranded DNA fragment with a single-strand nick in the presence or absence of poly(ADP-ribose) polymerase (PARP), which is a nick-sensor enzyme (Le Cam *et al.*, 1994; Cognet *et al.*, 1998).

This method is sensitive, as shown by the following observation. The goodness of the fit is checked with the computation of the reduced χ^2 in all cases except at high ratios ($r = 25$, 50), where the quality of the fit is marginal (cf. Figure 6). Therefore contributions of 10 to 20% of non-specific binding, as estimated from the gel electrophoresis experiments, are sufficient to distort and broaden significantly the original Gaussian distribution that is well observed at lower r values.

The use of two folded Gaussian distributions and of this method has allowed us to quantify the contributions of these distributions as a function of the concentration of free MC1. This gives directly the saturation ratio, which is the ratio of the concentrations, [DNA·MC1]/[DNA]. This is a macroscopic value obtained by collecting microscopic angle values and by statistical analysis.

Consistency of binding analysis by EM and gel electrophoresis

We have developed an original methodology to determine the mean angle value and the flexibility of the kink, and the dissociation constant of a DNA-binding protein with the simple measurements of angles obtained from EM images. To obtain this result, we have assumed that the ratio, r, is the same in solution and on the carbon film after adsorption and that the shape of the complex is unchanged after deposition on the EM grid.

The first assumption is verified by the gel electrophoresis experiment where DNA and MC1 are mixed at the same concentrations, at the same ratio and in the same buffer as for the EM experiments. Analysis of the gel bands corroborates the EM results with close K_d measurements within the experimental limits associated with the dilution of the initial salt concentration during the migration in the gel. The second assumption is corroborated by the internal coherence of the results. The population distributions of angles of double-stranded DNA have been analyzed in detail in the acompanying paper and are consistent with a two-dimensional (2D) random walk with practically no artefact within the approximately 20 bp resolution (Cognet *et al.*, 1998). In the presence of the MC1 protein, the angles follow Gaussian distributions at the different concentrations of MC1 used. Measurements of θ_a, σ_a, θ_b, σ_b, and of *aa* are well behaved in the regions where the binding is mostly specific at low MC1 concentrations. Deviation from the Gaussian distribution occurs at high MC1 concentrations, in agreement with the gel electrophoresis experiment which indicates binding by more than a single MC1 molecule. This is a marginal phenomenon but sufficient to perturb the measurements, as expected.

The equilibrium dissociation constant

The equilibrium dissociation constants for the non-preferential sites have been estimated to be on average 2000-fold higher than for the preferential site; this binding specificity is sufficient to perform DNAse I footprinting experiments (Teyssier *et al.*, 1994). However, as there are nearly as many non-specific potential binding sites as the number of nucleotides in the DNA fragment, there is a strong competition between the preferential binding site

and the non-specific binding sites. This non-specific binding is observed when r is in the range of 25 to 50 or higher, both by gel retardation and EM experiments. The K_d value obtained by gel electrophoresis is an estimated value which would have been difficult to obtain in the absence of the EM observations. As EM and gel experiments do cross-check in the same experimental conditions, the results presented here are a first reliable estimation of K_d for the single binding of MC1 at the preferential site.

The angle value of 116° and flexibility of the kink

We obtained the first clear demonstration that the binding of MC1 to DNA induces a large kink on linear fragments. The angle value found, 116°, is much larger than the value of ≈50° previously deduced from circular permutation analysis (Teyssier *et al.*, 1994). Electrophoretic methods are effective for the detection and localisation of a bend and its relative direction (Wu & Crothers, 1984; Zinkel & Crothers, 1987; Harrington, 1993) but can yield various and sometimes contradictory bend angle measurements (Lutter *et al.*, 1996; Kerppola & Curran, 1991; Gartenberg *et al.*, 1990; Kuprash *et al.*, 1995). Quantification of DNA bending remains controversial and depends on the methods used (Lutter *et al.*, 1996; van der Vliet & Verrijzer, 1993; Harrington, 1992; Kerppola, 1997). Even with X-ray crystallographic analysis the protein-induced DNA bend angle is known to be distorted by crystal packing forces (Lutter *et al.*, 1996). Values deduced from gel electrophoresis retardations should be considered as lower bounds for the reasons given previously. The reduction of gel electrophoresis migration is maximal only when the bend is located at the center. Because the binding site is only preferential, binding competition at sites neighboring the preferential site lowers the apparent kink angle. Therefore the value of 116° is the better determination.

The bend angle value of 116° is qualitatively in agreement with the capacity of MC1 to promote the circularisation of short DNA fragments of 110 bp by T4 DNA ligase (Laine *et al.*, 1991). It gives a precise measurement of the kinks previously observed on DNA minicircles by EM (Toulmé *et al.*, 1995), atomic force microscopy and cryo-EM (Larquet *et al.*, 1996). In the latter paper, we have shown that the 207 bp relaxed DNA minicircle is deformed upon binding by one MC1 molecule (complex 1) and even more by the formation of the complex 2, characterized by two diametrically opposed kinks resulting from the binding of two MC1 molecules. MC1 binding does not induce DNA wrapping around the protein, like the core of eucaryotic histones (Toulmé *et al.*, 1995). This deformation occurred out of the plane and suggested a torsion associated with the bending (Larquet *et al.*, 1996). Our results show that the flexibility of naked DNA at the preferential binding site is larger than in the presence of MC1. DNA flexibility plays an important role in the recognition processes by proteins and is reduced upon the formation of protein-DNA complexes (Grove *et al.*, 1996).

An increasing number of proteins are found to bend DNA upon binding to their recognition site, but very few of them have been described to induce sharp bends (Harrington, 1992; van der Vliet & Verrijzer, 1993; Werner *et al.*, 1996). MC1 is too small to be directly visualized by EM and to determine if it is localized inside or outside of the DNA curvature. The two sites hypersensitive to DNAse I and the relatively large segment of DNA that is protected from DNAse I would suggest that DNA wraps around MC1. DNA wrapping requires at least two distinct protein-DNA contacts. Proteins such as CAP, IHF, HU or TF1 also induce sharp bends, but they interact with DNA as hetero- or homodimers as opposed to MC1, which binds as a monomer. DNA bending can also occur by a widening of the minor groove outside of the DNA curvature or by kinking, which is defined as the unstacking of two base-pairs and an abrupt local change of the direction of the helical axis. The first mode, exemplified with DNAse I, induces limited bending (Suck & Oefner, 1986). The second mode, induced by minor groove intercalation, can sharply distort the DNA helix (Werner *et al.*, 1996) as for the high-mobility group (HMG) domain proteins HMG-1/2, SRY, LEF-1 and for the TATA binding proteins (TBP; Kim *et al.*, 1993). These proteins bind to DNA in monomeric form. This mode is usually associated with local unwinding of the DNA double helix. Owing to the binding of MC1 in a monomeric form, the induced sharp kink associated with unwinding of the double helix suggests that MC1 could distort DNA in a similar manner.

Conclusion

The characterization of the binding of MC1 to DNA achieved here is unusual in many respects. The study of the interaction of a histone-like protein, which is unspecific, would have been very difficult, if not impossible, without the preparation of these 176 bp fragments with a preferential site located at the center. In these fragments, the bends are so well defined that they can be studied as a function of MC1 concentrations. These favorable experimental features have authorized a novel, detailed analysis of the results whereby the information on the bends contained in the thousands of collected contours of molecules can be summarized with a few numbers (K_d, θ_a, σ_a, θ_b, σ_b) that are shown to be statistically relevant to account for all the information. The combination of different methods, i.e. the preparation of short DNA fragments containing a single preferential site, the use of EM and of a quantitative and critical analysis to characterize such a DNA-histone-like protein inter-

action, should open the way to quantitative studies. With this approach we have shown that it is possible to quantitatively measure, by microscopy, the deformations of DNA induced by protein-DNA interactions that are important in the biology of regulation and control.

Materials and Methods

DNA fragments

The 203 bp DNA fragment containing the *lac* promoter-operator region was isolated from a plasmid derived from pBR322 by *Eco*RI restriction cleavage (Fuller *et al.*, 1982).

The 176 bp DNA fragment containing sequences upstream of the gene coding for the methyl CoM reductase was obtained by *Alu*I restriction cleavage of a 1.2 kb fragment cloned in pUC9 (Allmansberger *et al.*, 1988). The position of the preferential binding site described by Teyssier *et al.* (1994) is located at the center of the fragment.

MC1 protein

Methanosarcina sp. CHTI 55 (DSM 2906) was grown as indicated by Chartier *et al.* (1988). Protein MC1 was prepared as previously described and the concentration determined by absorption spectrophotometry using an extinction coefficient of 11,000 $M^{-1}cm^{-1}$ at 280 nm.

Electron microscopy (EM)

Complexes were prepared by mixing MC1 protein and DNA linear fragments at different concentrations and different ratios of protein per DNA fragment in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl. At low MC1-DNA ratios, the complexes were directly deposited on the EM grids, whereas at high MC1/DNA ratios, complexes were first purified by gel filtration on a Superose 6B micro-column (Pharmacia), with a SMART system (Pharmacia). Note that high MC1/DNA ratios correspond also to high MC1 concentrations. EM observations were performed as described (Le Cam *et al.*, 1991; Le Cam & Delain, 1995). MC1 and DNA were incubated for 20 minutes at room temperature, and were observed by EM without any chemical fixation to stabilize the protein-DNA complexes. A 5 µl sample of the solution, containing 1 µg/ml of DNA, was deposited onto a 600 mesh copper grid coated with a very thin carbon film activated by a glow discharge in the presence of pentylamine according to Dubochet *et al.* (1971). Grids were washed with aqueous 2% (w/v) uranyl acetate, dried and observed in annular dark-field, in a LEO-Zeiss 902 electron microscope. Using this procedure, DNA molecules are rapidly adsorbed onto the carbon film with no major loss in the tridimensional information of their free conformation in solution (Joanicot & Révet, 1987). Individual molecules were directly analyzed at a final magnification of 560,000× on a Kontron image analysis system connected to the microscope through a video camera, as described (Le Cam *et al.*, 1991). Contour length measurements of the DNA and angle values were done by using the (x, y) coordinates obtained by digitization of the DNA images.

Computation of the bend

Angle-value analysis was performed as described (Le Cam *et al.*, 1994) and developed in the accompanying paper (Cognet *et al.*, 1998). To avoid taking into account local deformations or global deformations, only kinks with adjacent sides longer than 50 bp were taken.

Polyacrylamide gel electrophoresis (PAGE)

Complexes were prepared by mixing 5′ end-labelled DNA fragments (8.6 nM) and MC1 protein, at different ratios of protein per DNA fragment, in the same binding buffer conditions as above (10 mM Tris-HCl (pH 7.5), 50 mM NaCl). After equilibration for 20 minutes at 20 °C, samples were loaded onto a 5% polyacrylamide gel (acrylamide to bis-acrylamide, 19:1, w/w) in TBE buffer (0.09 M Tris base (pH 8), 0.09 M boric acid, 2 mM EDTA and subjected to electrophoresis at 10 V/cm for four hours at 20 °C. The gels were dried and autoradiographed with CGR films. The relative amount of DNA in each band was quantified with a PhosphorImager (Molecular Dynamics).

Computation of the equilibrium dissociation constant K_d

In gel electrophoresis or EM binding experiments, the total initial concentrations of MC1, $[MC1]_T$ and of DNA fragments, $[DNA]_T$, were assigned and the fractions of the DNA molecules with bound MC1, Y, were measured. To obtain the single-fit parameter K_d, the data were fitted to either of the two equations derived from a simple two-state binding process :

$$Y = \frac{[MC1 \cdot DNA]}{[DNA]_T} = \frac{[MC1]_T + [DNA]_T + Kd - \sqrt{([MC1]_1 + [DNA]_T + K_d)^2 - 4[MC1]_T[DNA]_T}}{2[DNA]_T}$$

This equation allows us to fit directly Y as a function of the experimentally assigned concentrations, $[MC1]_T$, and $[DNA]_T$:

$$Y = \frac{[MC1 \cdot DNA]}{[DNA]_T} = \frac{[MC1]}{K_d[MC1]}$$

This classical equation gives Y as a function of free [MC1], which can be computed from $[MC1]_T$ and $[MC1 \cdot DNA]$. Both treatments are equivalent and give the same K_d values.

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